Maximizing enzymatic hydrolysis efficiency of bamboo with a mild ethanol-assistant alkaline peroxide pretreatment

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Abstract

To overcome the delignification saturation point in traditional alkaline hydrogen peroxide pretreatment (AHP), a powerful modified AHP delignification methodology was established by introducing ethanol into the system. The pretreatment caused significant lignin removal of bamboo at elevated pretreatment temperature with the highest lignin removal reaching 80.0% at 100 °C, higher than that (74.9% lignin removal) in pretreatment without ethanol assistance.

In addition, a certain amount of carbohydrates was also solubilized during the process whose recovery was 83.3% (glucan) and 67.6% (hemicellulose), respectively. The pretreated solid exhibited excellent enzymatic digestibility, with hydrolysis yields of ~100% and 95.7% for glucan and xylan, respectively. Our studies further indicate that this delignification methodology is versatile for hardwood and herbaceous plants, but does not perform well with softwood.

Keyword: bamboo, low-temperature pretreatment, alkaline hydrogen peroxide， delignification, enzymatic hydrolysis

1. Introduction

The automobile market has grown substantially over the last decade as developing nations have developed their GDPs and accompanying this prosperity is the need for additional secure petroleum resources while addressing environmental challenges. Developing renewable fuels from lignocellulosic biomass offers a feasible approach to mitigating the issue of the finite petroleum resources, targeting engines that can utilize biofuels solely or as an additive, thereby reducing our dependence on the gasoline (Ragauskas et al., 2006). Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin, in which the carbohydrates of cellulose and hemicellulose can be depolymerized into monomeric sugars and biologically converted into fuels such as ethanol, butanol, and others (Huang et al., 2016). However, this process is hindered by several biomass natural properties, including the crystalline morphology of cellulose, covalent linkages between carbohydrates and lignin, and lignin's enwrapping effects on carbohydrates, forming a rigid and compact cell wall structure that restricts the accessibility of polysaccharides for degradation *via* enzymes (Mittal et al., 2017).

Pretreatments are thus proposed as an essential step in the lignocellulose biorefinery, which are targeted to break down the cell wall structures, thereby reducing biomass recalcitrance, and enabling the access of the carbohydrates to the enzymes. Thermochemical pretreatments are currently the leading pretreatment technologies, compared to the physical and biological pretreatments, in terms of the short reaction time and low energy consumption (Talebnia et al., 2010). These pretreatments are accomplished by introducing various chemicals into the cooking system, including acid, alkali, sulfite, oxidizing agents, and so on, that can remove hemicellulose and/or lignin, and improve the subsequent cellulose enzymatic hydrolysis efficiency (Meng & Ragauskas, 2014).

As one of the main factors responsible for biomass recalcitrance, lignin impedes the enzymatic hydrolysis by physically blocking the access of enzymes to the carbohydrates and by inhibiting cellulase activity (Lai et al., 2017). Thus, many of the pretreatments mentioned above are aimed to remove lignin. Nevertheless, the effects of a pretreatment on lignin in different biomasses can be varied, and this renders the lack of a versatile pretreatment technology to all the plant species. This effect is because of the lignin's heterogeneity, including the lignin content, distribution, and chemical structure, which are highly dynamic in nature and vary significantly from biomass to biomass, resulting in diverse biomass resistance to pretreatment and enzymatic hydrolysis. It is well known that lignin is typically composed of up to three basic units: guaiacyl (G), *p*-hydroxyphenyl (H) and syringyl units (S), and are mainly linked by *β-O-4*, *β-5* and *β-β* linkages (Hu et al. 2011). In addition, lignin is partially crosslinked with hemicellulose, forming a complex structure named lignin-carbohydrates complex (LCC). Biomasses differ in their contents of G, H, and S units and their inter-unit

67 linkages, and this makes plants exhibit different recalcitrance. For example, the G unit is the
68 dominant lignin monomeric fraction in gymnosperms, softwood lignins (with a minor amount
69 of H unit), whereas angiosperm dicot, hardwood lignins are composed of both G and S units. In
70 the case of the herbaceous plant, G and S units are at comparable levels, accompanied by an
71 elevated amount of H unit (Vanholme et al., 2010).

72 Alkaline pretreatment has long been regarded as a preferential process to cause biomass
73 delignification, as well as partial extraction of hemicelluloses, resulting in a substantial
74 increase of cellulose accessibility. However, traditional alkaline pretreatment is conducted
75 either at high temperature (140-200 °C) or with very long residence time (more than 12 h),
76 which are relatively energy-consuming and inefficient (Carvalho et al., 2016). Following
77 well-established bleaching procedures in the pulp and paper industry (Zeronian and Inglesby,
78 1995), this study proposed a novel ethanol-assistant alkaline peroxide pretreatment to enhance
79 lignin removal compared to aqueous $NaOH/H_2O_2$ pretreatment, and thus maximize the
80 enzymatic hydrolysis efficiency of a wide range of biomasses at a mild pretreatment
81 temperature of 100 °C for 60 min.

82 **2. Materials and methods**

83 2.1. Materials

84 Bamboo (*Neosinocalamus affinis*) culms were kindly provided by a pulping mill in
85 Guizhou province, China. The bamboo culms were first soaked in tap water overnight and then
86 subjected to a twin-screw extruder to cause fibrillation. Cellulase (*Cellic® CTec2*, Novozymes)
87 and xylanase (X2753-50g) were purchased from Sigma-Aldrich (Shanghai, China). All other
88 chemicals, of analytical grade, were provided by Sinopharm Chemicals Reagents Co. Ltd

89 (Beijing, China) and were used as received.

90 2.2. Biomass pretreatment

91 The pretreatment was conducted at a solid to liquid ratio of 1:10 in a 350 mL high-voltage
92 insulation glass flask. Modifying from a traditional oxygen bleaching process (Mussatto et al.,
93 2008), NaOH (in 20% solution), Na_2SiO_3 (in 10% solution), DTPA (in 5% solution), ethanol
94 (absolute ethyl alcohol) and DI water were mixed in a beaker to render the final NaOH,
95 Na_2SiO_3, DTPA and ethanol concentrations of 2.2wt%, 0.4wt%, 0.1wt%, and 15wt%,
96 respectively, which were the optimized loadings based on our previous research (Liang et al.,
97 2019). In the control experiment without ethanol (traditional AHP), DI water was used instead.
98 A certain amount of H_2O_2 (in 10% solution) was further added into the mixture mentioned
99 above, whose final concentration was 3 wt% in the pretreatment system. Next, the mixture was
100 poured into a beaker containing 20 g dry weight of biomass (all the chemicals concentrations
101 were calculated by accounting the initial water content in the biomass) and then vigorously
102 agitated for 2 min. The mixture was then transferred into the glass flasks and sealed with a
103 screw cap. Finally, the hermetic system was immersed in the hot water bath (with temperature
104 ranging from RT to 100 °C) and maintained for 60 min at the target temperature. After
105 pretreatment, the flasks were quenched in a cool water bath and then gently unscrewed the cap
106 to release the gases. The pretreated solid was washed with DI water until the rinsate was pH
107 neutral and the solids were then stored at 4 °C before use.

108 2.3. Enzymatic hydrolysis

109 Enzymatic hydrolysis was carried out in glass flasks with a working volume of 20 mL and
110 a consistency of 5%. Briefly, 1.0 g dry weight of pretreated solid was loaded into the flask,

111 followed by 2.0 mL 1 M acetate buffer to control the system pH around 4.8. Then cellulase and
112 xylanase were added with loadings of 25 FPU/g-glucan and 150 U/g-xylan, respectively.
113 Finally, DI water was supplemented to make the enzymatic hydrolysis system 20.0 mL.
114 Tetracycline (0.10 g/L) was used in all the runs to inhibit the microbial contamination. The
115 flasks were then placed in the 50 °C and 150 rpm orbital shaker and incubated for 72 h. Upon
116 the completion of enzymatic hydrolysis, 1 mL of the sample was withdrawn, centrifuged, and
117 then diluted for the HPLC analysis of glucose and xylose. All tests were carried out in
118 duplicate, and the results represented an average value.

119 2.4. Analytical procedure

120 The morphology of the raw and pretreated bamboo was observed with a Hitachi SEM
121 (Hitachi 3400-I, Japan) at an accelerating voltage of 15 kV. Before SEM test, samples were
122 oven-dried at 70 °C overnight and then sputter coated with gold. The crystalline structure of
123 the samples was analyzed by the XRD using a D8 advanced instrument (Bruker, Germany).
124 The XRD test was carried out using Cu Kα as X-ray radiation at a voltage of 40 kV and a
125 current of 30 mA. The recorded region of 2θ was from 10° to 40° with the scanning speed of
126 2°/min. FTIR spectra were obtained with a Nicolet 6700 spectrometer (Thermo Scientific, MA,
127 USA). The data was acquired in transmittance mode with 16 scans at 4 cm^{-1} resolution over the
128 range of 4000-600 cm^{-1}.

129 Chemical compositions of glucan, hemicellulose (xylan+arabinan), and lignin
130 (acid-soluble and acid-insoluble lignins) in all the samples were determined by a two-step
131 sulfuric acid hydrolysis procedure as reported by NREL (National Renewable Energy
132 Laboratory) (Sluiter et al., 2012). All the monomeric sugars in this study were quantified with

133 a HPLC (high-performance liquid chromatography, 1260 series, Agilent Technologies, Santa
134 Clara, CA, USA). The HPLC is equipped with a Bio-Rad Aminex PHX-87H column using
135 5mM H_2SO_4 as eluent at a flowing rate of 0.6 mL/min. Signals were detected with a refractive
136 index detector at 55 °C.

137 The following equations calculated enzymatic hydrolysis yield of glucan and xylan:

$$\text{Enzymatic hydrolysis yield (\%)} = \frac{\text{glucose or xylose in enzymatic hydrolyzate (g)}}{\text{initial glucose or xylose in substrate (g)}} \times 100\%$$

138

139 **3. Results and discussion**

140 3.1. Effect of pretreatment on the composition recovery

141 Lignin is generally regarded as an undesired component that hinders the degradation of
142 the carbohydrates by its crosslinking and blocking effects. In this study, bamboo
143 (*Neosinocalamus affinis*) was investigated as a typical recalcitrant biomass with a compact cell
144 structure and a highly lignified chemical structure. The raw bamboo contains 44.2% glucan,
145 19.5% hemicellulose (xylan+arabinan), 26.1% lignin (acid-soluble lignin+acid insoluble lignin)
146 and a small amount of other compounds of 1.9% ash and 5.9% benzene-ethanol extractives.
147 We adopted a bleaching-inspired ethanol-assistant AHP process to pretreat the bamboo in an
148 attempt to remove the lignin substantially and improve its enzymatic digestibility. The main
149 composition recovery after the pretreatment at different temperatures is shown in Fig. 1, and it
150 is compared with the traditional AHP process without ethanol assistance. It can be found that
151 both the pretreatments with and without ethanol could cause significant lignin removal,
152 especially at high temperatures. Specifically, lignin removal was increased from 36.4% to
153 46.7%, 63.5%, and 74.6%, respectively, as elevating temperature from RT to 40, 60, and 80 °C
154 (without ethanol, Fig. 1a). This result is in agreement with the previous report that NaOH and

155 H_2O_2 can degrade lignin by cleaving the lignin inter units linkages and causing the
156 ring-opening reaction (Huang et al., 2018). However, further increasing the pretreatment
157 temperature to 100 °C led to no apparent increase in lignin removal (74.9% vs 74.6%). Yuan et
158 al. also reported the same phenomenon, and the authors ascribed it to the existence of a lignin
159 degradation saturation point during the AHP (Yuan et al., 2018), which indicated that the
160 ~75% lignin removal was the saturation point in this study using traditional AHP pretreatment.
161 Whereas, when incorporating ethanol into the pretreatment system, delignification was
162 further reinforced, with the highest lignin removal reaching 80.0% at 100 °C (Fig. 1b),
163 suggesting that the so-called delignification saturation point could be overcome with the
164 introduction of ethanol. A plausible explanation of this breakthrough is that ethanol at alkaline
165 conditions provides a more powerful nucleophile than sodium hydroxide that could cause more
166 profound lignin degradation. In addition, it can be found that a certain amount of carbohydrates
167 was also removed during the pretreatment in which the removal of hemicellulose was more
168 pronounced than that of glucan. The carbohydrate degradation was probably caused by
169 oxidation reactions induced by the H_2O_2, and the peeling reaction under the alkaline
170 environment (Fernández-Delgado et al., 2019).
171 The results mentioned above verified the effect of our pretreatment in removing lignin. As
172 a result of the oxidative pretreatment chemistry, this delignification process whitened the
173 bamboo samples, even at RT with low lignin removal. Hydrogen peroxide at high pH forms the
174 perhydroxyl anions, a nucleophile that could oxidize the conjugated double bonds of
175 chromophores in lignin through the cleavage of the side chains, and thus decolorize the
176 substrate (Zeronian & Inglesby, 1995; Fukuzumi et al., 2012). A typical example proposed by

177 the authors is the transformation of the quinoid chromophore to the benzenoid structure under
178 the alkaline peroxide environment. Furthermore, it can be found that the bamboo brightness
179 was slightly improved with increasing the pretreatment temperature. In addition, when ethanol
180 was introduced into the system, bamboo brightness was further enhanced. These results are
181 consistent with the delignification results, indicating both high temperature and ethanol
182 introduction contributed to the lignin removal.

183 SEM was further used to investigate the effect of the pretreatment on the
184 micromorphology of the bamboo. The raw bamboo sample is composed of many long fibrils,
185 and some fibril bundles are clearly observed. However, these bundles are totally dissociated
186 after the AHP pretreatment with/without ethanol. In addition, it can be found that the long
187 bamboo fibrils tend to be shortened as elevating the pretreatment temperature from RT to 100
188 °C. As to the single bamboo fibril, the raw bamboo fibril had a smooth and compact surface
189 structure, while a wrinkled texture appeared after the bamboo was subjected to AHP
190 pretreatment which were caused by the degradation of large amount of lignin. These results are
191 consistent with previous results that the removal of lignin may cause collapses and cracks of
192 the cell wall structure and thus increase the surface area of the samples (Nitsos et al., 2019).
193 Besides, no apparent difference was observed between the pretreated samples for the AHP with
194 and without ethanol.

195 3.2. Chemical and crystalline structure variations during the pretreatment

196 Bamboo cellulose crystalline structure during the pretreatment was analyzed with X-ray
197 diffraction. It can be seen that there was no obvious shift and generation of the diffraction
198 peaks during the AHP with/without ethanol. As reported by Ling et al., significant transition of

cellulose I to cellulose II could be observed during the NaOH pretreatment at severe conditions (Ling et al., 2017), which indicated the proposed mild ethanol-assistant AHP showed no apparent effects on the cellulose structure. In addition, the CrI of the raw bamboo was 57.2%, higher than that of other biomasses, such as poplar and wheat straw (Wu et al., 2018). After the traditional AHP, the CrI was increased, with highest value of 66.7% at 80 °C. This is because of the removal of amorphous hemicellulose and lignin, leading to the increase of the content of crystalline cellulose in pretreated bamboo. However, further increasing pretreatment temperature to 100 °C decreased the CrI to 64.3%. The same phenomenon was also observed in samples pretreated with ethanol assistance, which was probably caused by the partial degradation of crystalline cellulose at high pretreatment temperature.

Moreover, FTIR spectra were used to analyze the chemical structure changes of the bamboo before and after the pretreatment. Characteristic peaks related to cellulose at 895 cm^{-1} (β-glycosidic linkages), 1034 cm^{-1} (C-O-C bonds associated with the pyranose ring skeletal vibration), 2893 cm^{-1} (C-H stretching) and 3331 cm^{-1} (O-H stretching) became intensive after the bamboo being subjected to the AHP, either with or without ethanol assistance (Zhang et al., 2019). These results are attributed to the removal of hemicellulose and lignin that increased the cellulose content in the pretreated bamboo samples. In addition, peaks at 1730 cm^{-1} and 1243 cm^{-1}, which are assigned to the C=O vibrations of acetyl group and ester stretching between hemicellulose and lignin, were significantly weakened after pretreatment, indicating partial degradation of hemicellulose in the AHP pretreatment (Qing et al., 2016). Furthermore, the aromatic skeletal vibrations in lignin at 1597 cm^{-1}, 1507 cm^{-1} and 1457 cm^{-1} also reduced after traditional AHP, and almost disappeared after the AHP was assisted with ethanol. These results

confirmed the composition analysis data that our ethanol-assistant AHP outperformed traditional AHP in removing lignin, which could generate the substrate with increased cellulose accessibility to the enzymes.

3.3. Enzymatic hydrolysis of the bamboo

As the key driver to evaluate the pretreatment efficiency, enzymatic hydrolysis of the pretreated bamboo was conducted, and the results are shown in Fig. 2. As expected, the raw bamboo (without any thermochemical pretreatment) exhibited a poor glucan and xylan hydrolysis yield of 13.8% and 9.9% (not shown in Fig. 2), respectively. On the other hand, the effects of the AHP pretreatment on the enzymatic hydrolysis can be clearly observed, with the glucan and xylan hydrolysis yields of 51.0% and 63.3%, respectively, at RT without ethanol introduction. The enzymatic hydrolysis efficiency was found to be significantly enhanced when increasing the pretreatment temperature. In detail, the glucan hydrolysis yield was increased from 51.0% to 58.2%, 76.7%, and 82.2%, respectively, with pretreatment temperature increasing from RT to 40 °C, 60 °C, and 80 °C, and plateaued thereafter (84.5% at 100 °C). The same trend was also observed for xylan hydrolysis yield, which increased from 63.3% to 72.2%, 79.7%, 87.1%, and 91.5%, respectively. The results mentioned above confirmed lignin as a significant obstacle hindering the enzymatic digestion of the bamboo; it is worth noting that, when the delignification reached the saturation point at 80 °C, enzymatic hydrolysis efficiency of the pretreated bamboo was also limited and cannot be further increased under the conditions studied.

Interestingly, when the pretreatment system included ethanol, not only was the delignification effect enhanced, but also the enzymatic hydrolysis yield was further improved,

with a maximum of ~100% for glucan and 95.7% for xylan obtained at a pretreatment temperature of 100 °C (Fig. 2b). The enzymatic hydrolysis results are in agreement with the lignin removal, validating that the introduction of ethanol could enhance bamboo delignification and thus improve its enzymatic hydrolysis efficiency. Whereas traditional delignification pretreatment were accomplished at severe conditions (high temperature and long residence time), some were even done using various solvents as the cooking media (such as ethanol and phosphoric acid), our ethanol-assistant AHP system delignified bamboo at only 100 °C for 60 min with low chemicals loadings, and thus maximized its enzymatic hydrolysis efficiency, outperforming that from pretreatments using either ethanol or other solvents as pretreatment liquid (Li et al., 2012; Sathitsuksanoh et al., 2010).

3.4. Versatility of the delignification system

To verify our delignification system as a versatile pretreatment method which is applicable to various lignocellulosic biomasses, five typical biomasses including hardwood (Poplar (*populous sp.*)), softwood (redwood (*metasequoia glyptostroboides*) and masson pine (*pinus massoniana*)) and herbaceous plants (wheat straw and reed (*phragmites australis*)) were subjected to our pretreatment. The enzymatic hydrolysis yields of the pretreated samples are shown in Fig. 3. It can be found that the pretreatment is particularly efficient for herbaceous plants of wheat straw and reed, which reached 100% glucan hydrolysis yield, even under the pretreatment without ethanol assistance. As to the poplar (hardwood), the pretreated sample could still exhibit a glucan hydrolysis yield as high as 91.9% (without ethanol assistance), and that increased to 95.7% with the ethanol-assistant delignification system. These results highlighted that the proposed pretreatment outperforms traditional thermochemical

pretreatment on improving the conversion of the carbohydrates for herbaceous plants and hardwood, especially when considering the low pretreatment temperature. However, in cases of the softwood, the delignification system does not perform well, especially for redwood. Specifically, masson pine glucan hydrolysis yield is 62.5% (without ethanol) that increased to 72.2% (pretreatment with ethanol). Nevertheless, only 22.2% (without ethanol) and 26.8% (with ethanol) glucan hydrolysis yields were reached for redwood. This phenomenon is possibly caused by the softwood lignin's feature of containing dominant G units and relatively high amount of highly stable *5-5* and *β-5* linkages that have shown stronger recalcitrance to our delignification system than hardwood and grass (Ralph et al., 2019). Further studies will be conducted to unveil the lignin structure changes of different biomasses in the pretreatment process.

4. Conclusion

The introduction of ethanol overcame the delignification saturation point in traditional AHP pretreatment of bamboo. Increasing pretreatment temperature from RT to 100 °C contributed to the pretreatment efficiency, with the highest lignin removal of 80.0% obtained at 100 °C, higher than that in pretreatment without ethanol (74.9% lignin removal). The pretreated bamboo is very reactive to enzymatic digestion, which showed hydrolysis yield of ~100% (glucan) and 95.7% (xylan), outperforming that without ethanol assistance (84.5% and 91.5% hydrolysis yield for glucan and xylan). Furthermore, our delignification system showed somewhat versatility on both hardwood and herbaceous plant, but are inefficient for softwood.

E-supplementary data for this work can be found in e-version of this paper online

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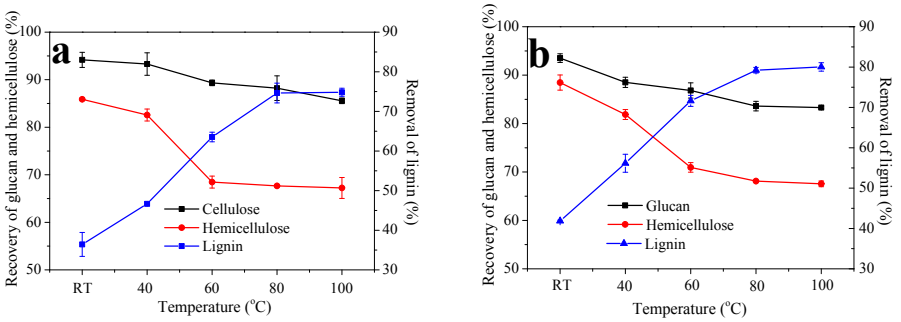
Figures Legends

Fig. 1. Main composition variation during the delignification system with (b) and without ethanol (a).

Fig. 2. Enzymatic hydrolysis yields of the bamboo pretreated at different temperatures.

Fig. 3. Enzymatic hydrolysis efficiency of different biomasses after pretreatment with the delignification system.



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373 **Fig. 1.**

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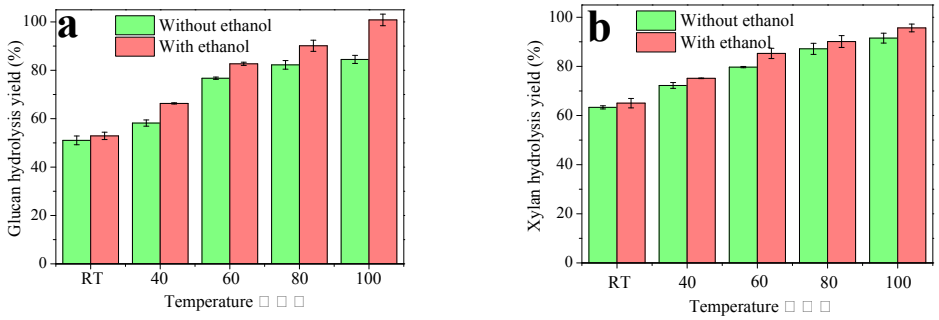

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377 **Fig. 2.**

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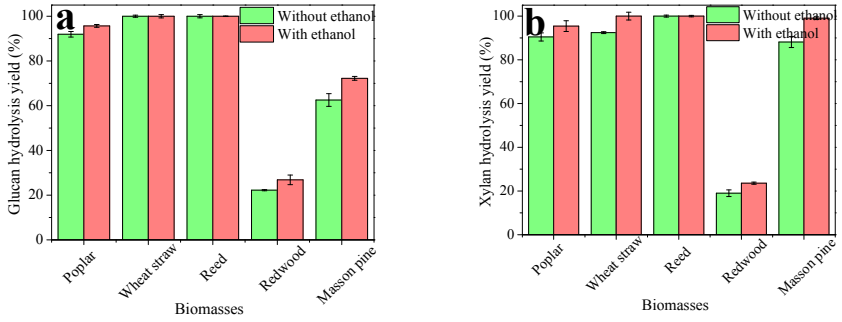

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381 **Fig. 3.**